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Antibacterial effects of cyanogenic glucoside isolated from the stem bark of *Bauhinia rufescens* Lam

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ABSTRACT

The chemical contents and *in vitro* antibacterial effects of the n-butanol column fractions, stem bark methanol extracts of *Bauhinia rufescens* Lam. were evaluated in Gram-positive and Gram-negative bacteria using disc diffusion technique. The n-butanol soluble portion from the stem bark methanol extract was successively eluted to afford 10 pooled fractions by comparison of TLC chromatograms. The bioactive fraction (fraction D) was further chromatographed using the bioassay guided protocol to afford 4 sub-fractions from which a pure compound was isolated and characterized using spectral studies as: (4*S*, 6*R*)-(*Z*)-6-(β-D-glucopyranosyloxy)-4-hydroxy-2-cyclohexenyl-$\Delta^{1,\alpha}$-acetonitrile–a *cyclohexenyl cyano methylene* known commonly as menisdaurin. Test bacteria were *Bacillus subtilis*, *Corynaebacterium* spp., *Escherichia coli* and *Shigella dysenteriae*. The diameters of inhibition zone on the Gram-positive bacteria was found in the range from11.83±0.17 (n-butanol Fraction D) to 17.33±0.67 (Fraction D2-8); while on Gram-negative organism the values ranged from 11.50±0.29 (n-butanol Fraction D) to 19.67±0.33 (Fraction D2-8). The activity index (AI) showed that Fraction D2-8 (51.18%) and Compound I [menisdaurin] (127.36%) was highly sensitive to *Corynaebacterium* spp. and *E. coli* when computed with Erythromycin and Gentamicin respectively. Highest AI was exhibited by Fraction D2-8 (93.89%) against *S. dysenteriae* when computed with ciprofloxacin. In continuation of our chemical studies and search for bioactive compounds from medicinal plants; this work, therefore, was aimed at characterizing and testing the antibacterial efficacy of the isolated compound in order to further confirm our earlier reports on use of this part of *B. rufescens* in some parts of Northern Nigeria as a treatment against diarrhoea and dysentery. This is the first report of the isolation and antibacterial activity of menisdaurin from *B. rufescens*.

Keywords*: Antibacterial, Bauhinia rufescens, cyanogenic glucoside, isolation, menisdaurin.*

INTRODUCTION

Medicinal plants, being the reservoir for drug and lead compounds for many therapeutic agents, are relatively safer and more affordable than synthetic alternatives (Akah et al., 2007; Iwu et al., 1999). There is global resurgence in the use of herbal preparations especially in some developing countries like Nigeria. These preparations are gradually sandwich into the primary and secondary health care delivery systems (El-Mahmood and Ameh, 2007). It has been estimated that more than 50% of Western drugs originated from plant materials

(Robbers et al., 1996). Several plants are indicated in folk and other traditional systems of medicines as anti-infective agents (El-Mahmood and Ameh, 2007). In recent times, there have been increases in antibiotic resistant strains of clinically important pathogens, which have led to the emergence of new bacterial strains that are multi-resistant (WHO, 2001, Aibinu et al., 2003; Aibinu et al., 2004; Odunayo et al., 2007). The non-availability and high cost of new generation antibiotics with limited effective span have resulted in increase in morbidity and mortality among the populace (Williams, 2000). Therefore, it is pertinent to continue research on drugs from medicinal plant sources. *Bauhinia rufescens* Lam is a scandent shrub of the family Leguminosae sub-family Caesalpiniodeae (FAO-UNEP, 1983; Burkill, 1995); it is deciduous in the drier area and an evergreen in wetter areas. It has wide array of medicinal values; the stem bark is used as cure for diarrhoea, dysentery, leprosy and to reduce fever (Burkill, 1995). In continuation of our interest in chemical studies and search for bioactive compounds from medicinal plants and to further confirm our earlier work on the crude extracts and partitioned portions of the stem bark of *B. rufescens* (Usman et al., 2009a,b,c). This work, therefore, was aimed at isolating and characterizing bioactive compound using chromatographic/spectral studies and then testing its efficacy against test bacteria using the hole-in-plate disc diffusion technique.

MATERIALS AND METHODS
Collection and identification of plant

The plant material (voucher number - #003/2008; *Bauhinia rufescens* Lam) was collected from Gathla village (Long. 13° 31.369′E, Lat. 11° 00.562′N), Gwoza - Borno State, Nigeria in June, 2008. The herbarium specimen was identified by Prof. S.S Sanusi of Biological Science Department, and the voucher specimen was deposited at the Post graduate Research Laboratory, Department of Chemistry, University of Maiduguri, Nigeria

for reference. The stem bark of the plant was air-dried under shade and pulverised into fine powder.

Extraction of plant materials

The concentrated n-butanol fractionated portion obtained from the crude methanol stem bark extract of *B. rufescens* (Usman et al., 2009a), was kept at 4 °C till use. The n-butanol Fraction D was subjected to detailed purification protocol using the column chromatography utilizing silica gel as adsorbent and *in vitro* antimicrobial susceptiblity analysis evaluated accordingly.

Test bacteria

Test bacteria were: *Bacillus subtilis, Corynaebacterium* spp. *Escherichia coli* and *Shigella dysenteriae*. The standard antibiotics disc used were Ciprofloxacin (5 μg/disc); Erythromycin (5 μg/disc) and Gentamicin (10 μg/disc). These bacteria were clinical isolates obtained from the Department of Medical Microbiology and Department of Veterinary Medicine, University of Maiduguri, Maiduguri-Nigeria.

Antimicrobial susceptibility test

The n-butanol Fraction D portion and sub-fractions of the stem bark extract of *B. rufescens* was subjected to antimicrobial evaluation on two Gram-positive and two Gram-negative strains using the hole-in-plate disc diffusion technique as described by Forbes et al. (1990); Vlietinck et al. (1995); Usman et al. (2007). Our earlier report revealed that fraction D contains cardenolides, cardiac and cyanogenic glycosides, flavonoids, resins, tannins (Usman et al., 2012).

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The extracts were made in three different stock concentrations of 1.5625, 3.125 and 6.25 mg/ml by dissolving 0.015625, 0.03125 and 0.0625 g respectively into 10 ml each of 85% methanol in distilled water (v/v) – as vehicle. The bacteria were maintained on agar slants until use. The inocula were then prepared by subjecting the test bacteria in nutrient broth and incubated for 24 hours at 37 ^{o}C. One millilitre of the diluted cultures was inoculated into 19 ml sterile molten nutrient agar (48 ^{o}C) and poured into sterile Petri dishes. Afterwards, holes of 9 mm internal diameter were bored onto the solidified and inoculated nutrient agar plates using sterilized number VI cork borer. All the holes were filled with equal volumes of 0.1 ml of different concentration to afford 0.156, 0.3125, 0.625 mg/hole. Standard discs were placed on bacterial-inoculated nutrient agar plate; the extracts were allowed to diffuse into the agar for an hour. Thereafter, the plates were incubated overnight at 37 ^{o}C. The extract was independently tested in triplicate. Diameters of inhibition zones (DIZ) $\geq$ 10 mm exhibited by plant extracts were considered active (Usman et al., 2007).

Activity index (AI)

This was evaluated as 100 x diameters of inhibition zone of extract ÷ diameters of inhibition zone of the standard antibiotic (expressed as %) (Shahidi, 2004).

Percent actvitiy (PA)

This was calculated as 100 x number of susceptible strains to a specific extract ÷ total number of tested bacterial strains. This was expressed as % Gram positive, % Gram negative and %T as total activity against both Gram positive and Gram negative (Shahidi, 2004).

Bacterial susceptibility index (BSI)

This was calculated as 100 x number of extracts effective against each bacterial strain ÷ number of total samples (expressed as %) (Shahidi, 2004).

Average percent of bacterial susceptibility (APBS)

This was determined as sum of % activities (%G+, %G-, or %T) ÷ number of total samples, this represents overall susceptibility of each group of bacterial strain (Shahidi, 2004).

Spectral intensity index (SII)

This was determined as: Mean diameters of inhibition zones (mm) of all sensitive bacterial strains to a specific sample x %T ÷ 100 (Shahidi, 2004).

Statistical analysis

The statistical analysis involved the determination of mean differences among the zone of inhibition exhibited by the extracts against each organism and the standard antibiotics analysed using One-way ANOVA with Student-Newman-Keuls Multiple comparisons test performed using GraphPad InStat (GraphPad Software, 1998).

Chromatographic separation of the n-butanol portion

The crude methanol extract was partitioned with various organic solvents as mentioned earlier (Usman et al., 2009b). The most susceptible portion (n-butanol fraction) was subjected to column chromatographic separation which afforded 10 pooled fractions coded A-J as reported earlier (Usman et al., 2012). Fraction D was observed to be active and then subjected to further purification protocol.

Isolation of compound I

About 15.0 g of Sephadex LH-20 (Amersham Biosciences, Sweden) were packed manually into a column of 45 cm x 1.4 cm *i.d.* The sephadex LH-20 was allowed to swell in MeOH overnight and cautiously filled into the column using a glass funnel. The gel was then allowed to settle and pack for 24 hrs. The air bubbles were avoided and care was taken not to dry the column. About 1.5 g of n-butanol fraction D was mounted on the

already equilibrated sephadex-fixed column. A total of 18 sub-fractions were generated and pooled in four relative groups: FD1, FD2-8, FD9-11 and FD12-18. FD1 (35 mg) and FD2-8 (80 mg) showed potent antimicrobial activities and thus separated further for isolation of the active compound(s). Continuous re-purification of FD2-8 by repetitive sephadex column chromatography and PTLC revealed D2-8:1 (56 mg) as pure colourless crystalline plates; the M.P. was found to be 170-172 ^{o}C uncorrected. The isolated compound coded *'Compound I'* was characterized using various analytical techniques: {IR, UV-Vis, NMR [[1]HMNR, COSY, ^{13}CNMR, DEPT, NOESY, HMBC, HMQC]} and was soluble in methanol and ethanol, but insoluble in petroleum ether, benzene and chloroform.

RESULTS
Characterization of compound I
The structure of compound I was elucidated and characterized using various analytical techniques. The Infra-red (IR) spectrum reveal strong absorption band around 2200 cm^{-1}. The UV absorption maximum at 259.0 nm.

The HNMR spectrum showed diagnostic peak for glucose at 4.571 ppm, a series of doublet-like signals centered at 3.336, 3.394, 3.287, 3.663, 3.881 ppm were equally found; two doublets appeared at 6.289 and 6.202 ppm and also at 5.512 ppm. Other signals were found at 4.913, 4.371, 2.252 and 2.033 ppm. The COSY spectrum showed the H-H couplings; the proton at 4.371 ppm coupled to protons at 2.033 and 2.252 ppm among others.

The ^{13}CNMR spectrum of the compound I in methanol exhibited fourteen carbon atoms approximately equal in height with some having characteristic chemical shifts equivalence. Definitive peaks were observed at 36.14, 63.11, 65.42, 71.73, 72.66, 74.55, 78.02, 78.13, 96.86 ppm. Others downfield peaks were at 101.59, 118.20, 127.85, 140.65 and 157.26 ppm. The DEPT

spectrum revealed that peaks at 157.26 ppm and 118.20 ppm lacks proton, those at 36.14 ppm and 63.11 ppm were methylene groups while the other nine peaks bears one hydrogen each 71.73, 72.66, 74.55, 78.02, 78.13, 96.86, 101.59, 127.85 and 140.65 ppm.

The heteronuclear multiple bond quantum coherence (HMQC) spectra showed correlations between the proton at 5.512 ppm and the carbon at 157.26 ppm. This carbon also resonates with the peak at 4.913 ppm. The heteronuclear multiple bond coherence (HMBC) spectrum revealed strong long range-correlations between proton at 5.491 ppm and carbon at 127.85 ppm, this proton equally interacted with the carbon at 118.20 ppm. Weak interactions were noted between proton at 5.491 ppm with the carbon at 74.55 ppm as well as carbon at 157.26 ppm. The NOESY spectra showed that proton at 4.373 ppm interacted very well with proton at 6.202 ppm while the proton at 4.913 ppm showed interaction with that 4.571 ppm. Others were observed between 4.571 and 2.252 ppm; 2.033 ppm.

Bacterial susceptibility pattern of sub-fractions D and compound I
The results of the bacterial susceptibility pattern of the n-butanol column fraction D and its sub-fraction at the dose of 0.3125 mg/hole are presented in Table 1. The activities ranged from 11.83 ± 0.17 to 19.00 ± 0.00 among the organisms tested. Table 2 shows the susceptibility pattern of D2-8 and compound I. Least activity of 13.33 ± 0.33 was exhibited by sub-fraction D2-8 at concentration of 0.156 mg/hole against *B. subtilis* while the highest activity was expressed by the same sub-fraction but at 0.625 mg/hole against *S. dysenteriae*. The sensitivity indices namely the activity index, bacterial susceptibility index, spectral intensity index and average percent of bacterial susceptibility were shown on Table 3a, 3b.
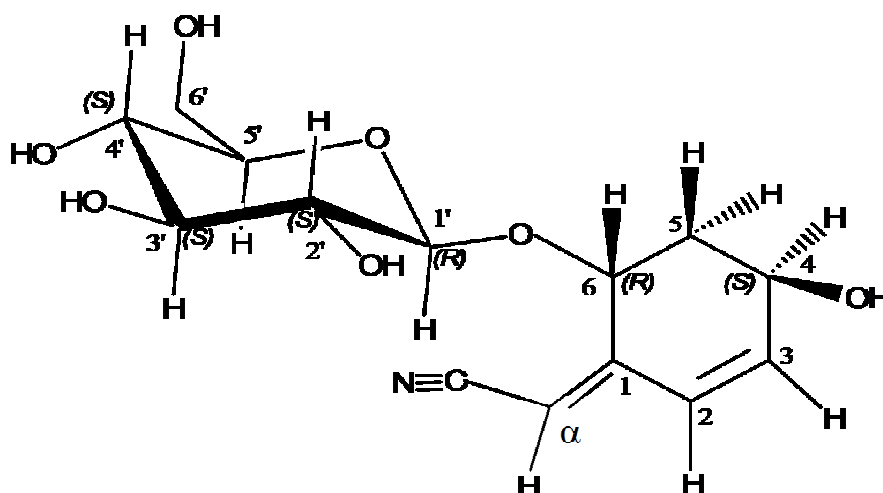
Figure 1: Compound I: (4*S*, 6*R*)-(Z)-6-(β-D-glucopyranosyloxy)-4-hydroxy-2-cyclohexenyl-$\Delta^{1,\alpha}$-acetonitrile–a *cyclohexenyl cyano methylene.*

Table 1: Effects of column fraction D and sub-fractions of the n-butanol portion of the stem bark of *B. rufescens* on some bacteria at 0.3125 mg/hole.

S/No	Fractions	Bacteria/diameters of Inhibition zone (mm)			
		B. subtilis	*Corynaeb.* spp.	*E. coli*	*S. dysenteriae*
1	NBD	11.83 ± 0.17^a	13.17 ± 0.17^a	11.50 ± 0.29^a	14.33 ± 0.17^a
2	D1	16.33 ± 0.33^b	16.67 ± 0.17^b	$15.33 \pm 0.33^{b,c}$	17.17 ± 0.60^b
3	D2-8	16.67 ± 0.33^b	16.33 ± 0.33^b	16.33 ± 0.33^b	19.00 ± 0.00^b
4	D9-11	15.67 ± 0.33^b	14.33 ± 0.33^b	17.67 ± 0.17^d	17.67 ± 0.67^b
5	D12-18	16.67 ± 0.88^b	15.33 ± 0.33^b	14.33 ± 0.33^b	17.67 ± 0.33^b
6	Ciprofloxacin (5 µg/disc)	25.33 ± 0.33^c	36.00 ± 1.00^c	26.67 ± 0.67^e	$20.00 \pm 0.00^{b,c}$
7	Erythromycin (5 µg/disc)	23.67 ± 0.67^c	33.00 ± 0.00^d	28.33 ± 0.33^f	$19.83 \pm 0.17^{b,c}$
8	Gentamicin (10 µg/disc)	24.33 ± 0.33^c	22.33 ± 1.36^e	14.00 ± 0.00^b	17.33 ± 0.67^b

Key: Means with different superscript along same column are statistically different (P<0.05); NBD = n-butanol column fraction D. Data are presented as mean ± SEM, n = 3.

Table 2: Effects of compound I and column fraction D2-8 of the n-butanol portion of the stem bark of *B. rufescens* on some bacteria at various doses.

S/No	Dose (mg/hole)	Bacteria/diameters of inhibition zone (mm)			
		B. subtilis	*Corynaeb. s*pp.	*E. coli*	*S. dysenteriae*
1	D2-8 (0.625)	$17.17 \pm 0.17^{a,b}$	16.67 ± 0.33^a	18.33 ± 0.33^a	19.67 ± 0.33^a
2	Compound I (0.625)	17.33 ± 0.67^b	15.33 ± 0.33^a	17.33 ± 0.33^a	17.33 ± 0.33^b
3	D2-8 (0.313)	$16.67 \pm 0.33^{b,c}$	16.33 ± 0.33^a	16.33 ± 0.33^b	19.00 ± 0.00^a
4	Compound I (0.313)	14.33 ± 0.33^c	16.83 ± 0.33^a	18.33 ± 0.33^a	14.33 ± 0.88^d
5	D2-8 (0.156)	13.33 ± 0.33^c	17.67 ± 0.33^a	14.67 ± 0.17^c	17.67 ± 0.17^c
6	Ciprofloxacin (5 µg/disc)	25.33 ± 0.33^d	36.00 ± 1.00^b	26.67 ± 0.67^d	20.00 ± 0.00^a
7	Erythromycin (5 µg/disc)	23.67 ± 0.67^d	33.00 ± 0.00^c	28.33 ± 0.33^e	19.83 ± 0.17^a
8	Gentamicin (10 µg/disc)	24.33 ± 0.33^d	22.33 ± 1.36^d	14.00 ± 0.00^c	17.33 ± 0.67^c

Key: Means with different superscript along same column are significantly different (P<0.05). Data are presented as mean ± SEM, n = 3

Table 3a: Activity and bacterial susceptibility indices of compound I, fraction D and sub-fractions of the n-butanol portion of the stem bark of *B. rufescens* on some bacteria.

	Activity index (%)							
Fractions	*B. subtilis*		*Coryneab.* spp.		*E. coli*		*S. dysenteriae*	
D	$^a60.76$	$^b65.02$	$^a41.36$	$^b45.12$	$^a54.14$	$^c103.14$	$^a75.30$	$^c86.90$
D1	$^a64.47$	$^b68.99$	$^a46.31$	$^b50.52$	$^a57.48$	$^c109.50$	$^a85.85$	$^c99.08$
D2-8	$^a62.07$	$^b66.41$	$^a46.91$	$^b51.18$	$^a61.03$	$^a116.29$	$^a93.89$	$^c108.37$
D9-11	$^a61.86$	$^b66.20$	$^a39.81$	$^b43.42$	$^a66.24$	$^c126.21$	$^a88.33$	$^c101.96$
D12-18	$^a65.81$	$^b70.43$	$^a42.58$	$^b46.45$	$^a53.73$	$^c102.36$	$^a88.35$	$^c101.96$
Compound I	$^a62.50$	$^b66.88$	$^a44.67$	$^b48.73$	$^a66.89$	$^c127.36$	$^a79.15$	$^c91.34$
*BSI (%)	79.17		87.50		79.17		91.67	

Key: Activity index when computed with: a=Ciprofloxacin; b=Erythromycin; c=Gentamicin; * ≥15mm (DIZ) as the susceptible value.

Table 3b: Percent activity and average percent of bacterial susceptibility of compound I, fraction D and sub-fractions of the n-butanol portion of the stem bark of *B. rufescens* on some bacteria.

Fractions	Percent Activity (%)			*Spectral Intensity Index	Average Percent of Bacterial Susceptibility (%)		
	G+ve	G-ve	%T		G+ve	G-ve	%T
D	96.30	92.60	94.45	14.24[a]	93.33	85.00	89.17
D1	100	100	100	14.60[b]			
D2-8	83.33	83.33	83.33	15.09[c]			
D9-11	100	100	100	14.57[b]			
D12-18	100	66.67	83.34	14.27[a]			
Compound I	83.33	75.00	79.17	14.62[b]			

Key: G+ve = Gram-positive; G-ve = Gram-negative; T= total, *values with different superscript along same column are statistically significant (P<0.05), * ≥15mm (DIZ) as the susceptible value.

DISCUSSION

The structure of compound I was elucidated and characterized using the analytical techniques. In structure elucidation of cyanogenic compounds, the Infra-red (IR) spectrum is a diagnostic tool that is expected to reveal strong nitrile absorption as a result of significant dipole moment associated with the CN bond leading to a significant change when it interacts with IR radiation usually appearing as an intense sharp peak at 2200- 2280 cm^{-1}. In fact, very few other groups absorb at this region with this intensity and also together with a C=C stretching vibrations at 1620 cm^{-1} (Eudea et al., 1983). The nitrile group is a reliable functional group that is generally easy to identify. From the IR spectrum, the following wave numbers were observed and assigned accordingly: 3359 cm^{-1} (-OH$_{(str. -HB)}$), 2954 cm^{-1}-CH$_2(v_{asy})$; 2845 cm^{-1}(-CH$_2(v_{sym})$; 2224 cm^{-1}(-CN); 1637 cm^{-1} (-C=C ($v_{str.}$); 1457 cm^{-1} (-CH$_{(bend[rocking])}$); 1424 cm^{-1} (-C=C$_{(bend [scis])}$); 1018 cm^{-1} (-C-O$_{(str)}$); (Takahashi et al., 1978; Seigler et al., 2005). Worthy of note is the broad band between 1099.8-1029.4 cm^{-1} indicative of the glycosidic character of the compound (Tang et al., 2003). The UV absorption maximum at 259.0 nm suggests the presence of αβγδ-unsaturated nitrile group (Dwuma-Bada et al., 1976; Takahashi et al., 1978; Seigler et al., 2005).

The HNMR spectrum confirms the presence of a glucosyl group in compound I as shown by the presence of a doublet signal at 4.571 ppm (*J*=7.5 Hz, H-1′), a series of doublet-like signals centered at 3.336, 3.394, 3.287, 3.663, 3.881 and 3.336 (H-5′, H-3′, H-4′, H-6′b, H-6′a, H-2′) are strong signals for sugar protons. The large overlap of double doublets between 3.306 and 3.336 ppm are indicative of glucose moiety. Two vinyl doublets appears at 6.289 (H-2) and 6.202 (H-3) ppm and additional vinyl proton (α-H) at 5.512 ppm. Two other protons are on carbon bearing hydrogen: doublet at 4.913 and 4.371 {H-6ax, 4ax}. The two geminally available protons appeared at 2.252 and 2.033 ppm {H-5eq and H-5ax} respectively corresponding to the methylene protons. The absence of singlet at δ3.70–3.90 ppm is indicative of the relative absence of methoxyl group, similarly no peak was observed between δ0.80-1.20 ppm characteristics of Rhamnose sugar (Mabry et al., 1970; Manguro et al., 2005) and hence the absence of these molecules. The COSY spectrum showed the H-H couplings; there is long range interactions observed between the protons, H-H coupling shows some definitive

information about the aglycone moiety of compound I. The unique coupling at 4.371 (H-4ax) is found coupled to up field protons at 2.033 and 2.252 ppm ({H-5ax and H-5eq} and equally to the three vinyl protons (H-α,2,3). The vinyl proton at 5.512 ppm is coupled to the two other vinyl protons as well as to the peaks centered at 4.913 and 4.371 ppm H-6ax, 4ax ppm which is line with earlier reports by Jaki et al. (2003) and Seigler et al. (2005). The multiplicities and couplings observed on the aglycone are as follows: α-H[5.512 ppm], *d,* $J_{\alpha\text{-}6}$=1.5 Hz; 2-H[6.287 ppm], *ddd,* $J_{3,2}$=10.0 Hz, $J_{2,4}$=1.0 Hz, $J_{2,\alpha}$=1.0 Hz, 3-H[6.202 ppm], *dd,* $J_{2,3}$=10.0 Hz, $J_{3,4}$=3.0 Hz; 4ax-H[4.371 ppm], *m,* J=12.0 Hz; 4eq-OH[4.753 ppm*{acetone}*], d*{broad}*, $J_{4\text{-}OH,4}$=6.2 Hz; 5eq-H[2.252 ppm], *ddd,* $J_{5,5}$=13.0 Hz, $J_{5,6}$=8.5Hz, $J_{5,4}$=3.5 Hz; 5ax-H[2.033ppm], *ddd,* $J_{5,5}$=13.5 Hz, $J_{5,6}$=8.0 Hz, $J_{5,4}$=6.0 Hz; 6ax[4.913 ppm], *ddd* $_{6,5ax}$=8.5 Hz, $J_{6,5eq}$=3.5 Hz, $J_{6,\alpha}$=1.5 Hz. The couplings due to sugar protons showed multiplicities: 1′-H[4.571 ppm], *d,*=7.5 Hz; 2′-H[3.336 ppm], *dd,* $J_{2',3'}$=9.0 Hz, $J_{2',1'}$=6.5; 2′-OH[4.956 ppm*{acetone}*], *dd,* $J_{2'\text{-}OH, 2'}$=3.0 Hz; 4′-H[3.287 ppm], *dd,* $J_{4',3'}$= $J_{4',5'}$=9.5 Hz; 4′-OH[4.939 ppm*{acetone}*], *d,* $J_{4'\text{-}OH,4'}$=3.0 Hz; 3′-H[3.394 ppm], *dd,* $J_{3',2'}$= $J_{3',4'}$=8.5 Hz; 3′-OH[4.945 ppm*{acetone}*], *d,* $J_{3'\text{-}OH,3'}$=2.5 Hz; 5′-H[3.336 ppm], *ddd,* $J_{5',4'}$=9.0 Hz, $J_{5',6a'}$=6.5 Hz, $J_{5',6b'}$=2.0 Hz; 6a′-H[3.663 ppm], *dd,* $J_{6a',6b'}$=12.0 Hz, $J_{6a',5'}$=6.0 Hz; 6b′-H[3.881 ppm], *dd,* $J_{6b',6a'}$=12.0 Hz, $J_{6b,'5'}$=2.0 Hz and 6′-OH[4.520 ppm*{acetone}*], d, $J_{6'\text{-}OH,6'b}$=11.5 Hz, $J_{6'\text{-}OH,6'a}$=8.0 Hz.

The ^{13}CNMR spectrum of the compound I in methanol exhibited fourteen carbon atoms approximately equal in height with some having characteristic chemical shifts equivalence. The presence of peak at 101.59 is typical of anomeric carbon of glucoside (C-1′); other doublet peaks at 78.02, 78.13, 72.66, 71.73 and 63.11 ppm supported the fact that the compound contained β-glucopyranose moiety (Pauli, 1993; Seigler et al., 2005) and the presence of singlet peak at

118.20 ppm is assignable to the $\alpha\beta$-unsaturated nitrile carbon (Eudea et al., 1983; Seigler et al., 2005). Other signals at 36.14 ppm (a methane or methylene carbon, C-5) 65.42 and 74.55 ppm (carbon bearing oxygen; C-4 and C-6 respectively) while the signal at 96.86, 127.85, 140.65 and 157.26 ppm (aromatic or vinyl carbons; α-C, C-3, C-2, C-1 respectively). The DEPT spectrum revealed that the peaks at 157.26 ppm (C-1) and 118.20 ppm (CN) lack protons, those at 36.14 ppm (C-5) and 63.11 ppm (C-6′) are methylene groups while the other nine carbons bear one hydrogen [α-C(96.86), C-2(140.65), C-3(127.85), C-6(74.55), C-1′(101.59), C-2′(72.66), C-3′(78.02) C-4′(71.73) and C-6′(78.13)].

The heteronuclear multiple bond quantum coherence (HMQC) spectra showed that there were interactions between the proton at 5.512 ppm (H-α) and the vinyl carbon at 157.26 ppm (C-1), the carbon of the proton resonating at 4.913 ppm (H-6), the nitrile carbon and the sugar anomeric carbon, suggest that these groups are proximal in the structure. Because the methylene group protons at 2.252 and 2.025 ppm (H-5eq, 5ax) are coupled both to the proton at 4.371 ppm (H-4ax) and that at 4.913 ppm (H-6), a six membered ring is required (Seigler et al., 2005). The heteronuclear multiple bond coherence (HMBC) spectrum revealed strong long range-correlations between proton at 5.491 ppm (H-α) and carbon at 127.85 ppm (C-2) suggestive of *cis*-relationship, the proton equally interacted with the nitrile carbon at 118.20 ppm suggesting that this proton and the nitrile group are attached to the same carbon atom. The signal 5.491 (H-α) also interacted weakly with the oxygen-bearing carbon at 74.55 ppm (C-6) and the vinyl carbon at 157.26 ppm (C-1). There is a strong interaction of this proton (H-α) with the anomeric carbon (C-1′) at 101.59 ppm which suggests that the site of attachment of the sugar is on the carbon at 74.55 ppm (C-6) (Takahashi et al., 1978; Seigler et al., 2005).

The NOESY experiment is designed to reveal the kind of protons that are closer in space showed that the proton at 4.373 ppm (H-4) has an interaction with the vinyl proton at 6.202 ppm (H-3) while the proton at 4.913 ppm (H-6) interacted favourably with the anomeric proton at 4.571 ppm (H-1′). Closer interactions between the anomeric proton {4.571 ppm (H-1′)} with the up field protons at 2.252 ppm (H-5eq) and also 2.033 ppm (H-5ax) were observed. The mass spectra showed base peak at 314.1243 $[M+H]^+$ while the glucose fragmentation appeared at M^+ 152.0715 when run with an internal standard {[Glu]-Fibrinopeptide B}. According to Takahashi et al. (1978), the mass spectra of the nitrogen containing compound mostly had an even numbered M+H value in electron impact mode and therefore suggestive of nitrogenous compound. Consistently, the accurate mass measurement of compound I showed M/Z peak at 313.1243 which is in a closer proximity to the isotopic theoretical value of M/Z peak 313.1240. Therefore, taken collectively the spectral information suggested a planar conjugated system with two double bonds, a nitrile group, a glucose moiety and an oxygen-bearing carbon at 4.371 ppm (H-4ax). Furthermore, the proton at 5.512 ppm (H-α) is in *cis*-relationship to one of the vinyl carbons. Based on the ^{13}CNMR and mass spectral data, the molecular formula requires six sites of unsaturation, hence a ring in both the sugar and the aglycone portion of the compound. Though, the structure of compound I was established earlier (Sosa et al., 1977; Takahashi et al., 1978; Nahrstedt and Wray, 1990; Seigler et al., 2005) as Menisdaurin; it is indeed, the first time the compound is run in acetone to confirm the OH-configurations on the compound in relation to the earlier use of the DMSO by Takahashi et al. (1978). In line with the newer technologies in structure elucidation (HMBC, HMQC, NOESY), the spectral data of the isolated compound as shown by the conformational and structural configuration as $C_{14}H_{19}NO_7$ conforms well with the compound's spectra data reported earlier by Seigler et al. (2005) as: (4*S*, 6*R*)-(*Z*)-6-(β-D-glucopyranosyloxy)-4-hydroxy-2-cyclohexenyl-$\Delta^{1,\alpha}$-acetonitrile–a *cyclohexenyl cyano methylene* structure shown in the Figure 1 below.

Menisdaurin was considered non-cyanogenic (Nahrsredt and Wray, 1990; Yamasaki, 1997) and was later noted to liberate small amount of cyanide by sandwich method of TLC (Seigler et al., 2005) and also under certain conditions there has been reports that cyanogenic glycosides with nitrile group alpha to the glycosidic linkage have been known to liberate HCN upon hydrolysis (Lechtenberg and Nahrstedt, 1999; Yamasaki, 1997). However, the mechanisms of cyanide liberation from Cyanogenic glycosides with nitrile group (example menisdaurin, ehretioside B) not adjacent to the glycosidic linkage still remain unknown (Yamasaki, 1997).

The susceptibility pattern shown on Table 1 revealed that wider diameters of inhibition zones (DIZ) of 16.67 mm were expressed against Gram-positive bacteria - *B. subtilis* (D2-8, D12-18) and *Corynaebacterium* spp. (D1). However, lower DIZ was found as 14.33 mm against *Corynaebacterium* spp. which was indeed higher than the value expressed by n-butanol portion at same dose (Usman et al., 2009b). The effects on Gram-negative bacteria were expressed by D2-8 with higher activity of 19.00 mm against *S. dysenteriae* while 14.33 mm was observed as the lowest DIZ expressed by D12-18 against *E. coli.*

The results on Table 2 below revealed high activity against *B. subtilis* at 0.625 mg/hole expressed by compound I; the least value of 14.33 mm was however exhibited by D2-8 against same bacteria.

The bacterial activities indices shown on Table 3a,b indicates high activity index of 65.81% and 70.43% when computed with Ciprofloxacin and Erythromycin against *B. subtilis* and the least value of 39.81% and 43.42% when computed similar antibiotics

against *Corynaebacterium* spp. More so, the AI against Gram-negative bacteria had higher percentage of 93.89% when computed with Ciprofloxacin and Gentamicin against *S. dysenteriae.* However, compound I had AI of 66.89% and 127.36% when computed with Ciprofloxacin and Gentamicin against *E. coli;* suggesting compound I being a better agent compared to Gentamicin against this notorious bacteria. The BSI indicated high percentage value of 91.67% against *S. dysenteriae* while 79.17% was lowest against *E. coli* and *B. subtilis.* The percentage activity varied from 66.67-100% against both Gram-positive and Gram-negative bacteria. The SII showed D2-8 having high value of 15.09 mm while compound I followed with a value of 14.62 mm, expressing the compound being very active (Usman et al., 2007), and the APBS was higher towards Gram-positive bacteria with 93.33%. This trend explains the efficacy of fraction D2-8 and compound I. These susceptibility patterns were similar to those reported earlier (Parekh et al., 2006; Usman et al., 2007; Geidam et al., 2007).

Although cyanogenesis was earlier reported in Fabaceae (Caesalpinioideae), this is the first report on the isolation and characterization of cyanogenic glucoside (menisdaurin) from the species *B. rufescens* as well as the bioassay directed antibacterial effects against some bacterial strains *in vitro.* This study therefore showed that menisdaurin was highly susceptible to *B. subtilis* and *S. dysenteriae.*

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